Immunity
Article

C-type Lectin MCL Is an FcRγ-Coupled Receptor that Mediates the Adjuvanticity of Mycobacterial Cord Factor

Yasunobu Miyake,[1,2,8] Kenji Toyonaga,[1,8] Daiki Mori,[1,8] Shigeru Kakuta,[5] Yoshihiko Hoshino,[6] Akiko Oyamada,[3] Hisakata Yamada,[2,3] Ken-ichiro Ono,[7] Mikita Suyama,[4] Yoichiro Iwakura,[5,9] Yasunobu Yoshikai,[2,3] and Sho Yamasaki[1,2,*]
[1]Division of Molecular Immunology
[2]Research Center for Advanced Immunology
[3]Division of Host Defense
[4]Division of Bioinformatics
Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-8582, Japan
[5]Department of Molecular Pathogenesis, Center for Experimental Medicine and Systems Biology, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan
[6]Leprosy Research Center, National Institute of Infectious Diseases, Tokyo 189-0002, Japan
[7]Research and Development Department, Ina Laboratory, Medical & Biological Laboratories Corporation, Ltd., Ina, Nagano 396-0002, Japan
[8]These authors contributed equally to this work
[9]Present address: Division of Laboratory Animal, Research Institute for Biomedical Sciences, Tokyo University of Science, Chiba 278-0022, Japan
*Correspondence: yamasaki@bioreg.kyushu-u.ac.jp
http://dx.doi.org/10.1016/j.immuni.2013.03.010

SUMMARY

Cord factor, also called trehalose-6,6′-dimycolate (TDM), is a potent mycobacterial adjuvant. We herein report that the C-type lectin MCL (also called Clec4d) is a TDM receptor that is likely to arise from gene duplication of Mincle (also called Clec4e). Mincle is known to be an inducible receptor recognizing TDM, whereas MCL was constitutively expressed in myeloid cells. To examine the contribution of MCL in response to TDM adjuvant, we generated MCL-deficient mice. TDM promoted innate immune responses, such as granuloma formation, which was severely impaired in MCL-deficient mice. TDM-induced acquired immune responses, such as experimental autoimmune encephalomyelitis (EAE), was almost completely dependent on MCL, but not Mincle. Furthermore, by generating *Clec4e*^{gfp} reporter mice, we found that MCL was also crucial for driving Mincle induction upon TDM stimulation. These results suggest that MCL is an FcRγ-coupled activating receptor that mediates the adjuvanticity of TDM.

INTRODUCTION

C-type lectin receptors (CLRs) constitute a large family of carbohydrate binding proteins that is highly conserved in vertebrates (Zelensky and Gready, 2005). CLRs have been recently identified as pattern recognition receptors (PRRs) for pathogens, similar to Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and NOD-like receptors (NLRs) (Hardison and Brown, 2012; Takeuchi and Akira, 2010). Some members of the CLR family utilize an immunoreceptor tyrosine-based activation motif (ITAM) to transduce activating signals possessed within their own cytoplasmic tails, or by coupling with ITAM-bearing signaling subunits, such as the Fc receptor γ (FcRγ) chain or DAP12 (Robinson et al., 2006).

We have recently found that the C-type lectin Mincle (macrophage inducible C-type lectin, also called Clec4e or Clecsf9) is an activating receptor that couples with the FcRγ chain and recognizes cord factor, a mycobacterial glycolipid (Ishikawa et al., 2009; Yamasaki et al., 2008). Cord factor is an immunostimulatory component that elicits pulmonary inflammation (Bloch, 1950; Yamaguchi et al., 1955). In 1956, the chemical structure of cord factor was established as trehalose-6,6′-dimycolate (TDM) (Noll et al., 1956). Mincle is an essential receptor for TDM-induced innate immune responses, such as granulomagenesis, as well as in vitro macrophage activation (Ishikawa et al., 2009; Schoenen et al., 2010). TDM induces the upregulation of Mincle itself (Lee et al., 2012; Schoenen et al., 2010). However, Mincle is barely detectable in resting cells. This prompted us to hypothesize that there was an alternative TDM receptor(s) that might be present in resting cells to drive the initial expression of Mincle.

MCL (macrophage C-type lectin, also called Clec4d or Clecsf8) was first cloned as a type II transmembrane C-type lectin receptor that is expressed in myeloid cells (Arce et al., 2004; Balch et al., 1998). It has been recently proposed that MCL acts as an activating receptor (Graham et al., 2012). However, the signaling subunit and ligand of MCL remains unidentified. MCL is located on murine chromosome 6 (6F3) and human chromosome 12 (12p13), in which activating C-type lectin receptors, including Dectin-1, Dectin-2, and Mincle, are clustered (Arce et al., 2004; Flornes et al., 2004). Dectin-1 is a myeloid-specific β-glucan receptor that signals through a hemITAM within its own cytoplasmic tail (Rogers et al., 2005). Dectin-2 is associated with FcRγ and recognizes high-mannose oligosaccharides of fungi (Saijo et al., 2010; Sato et al., 2006). Mincle is also an FcRγ-coupled activating receptor that recognizes pathogens such as fungi or mycobacteria

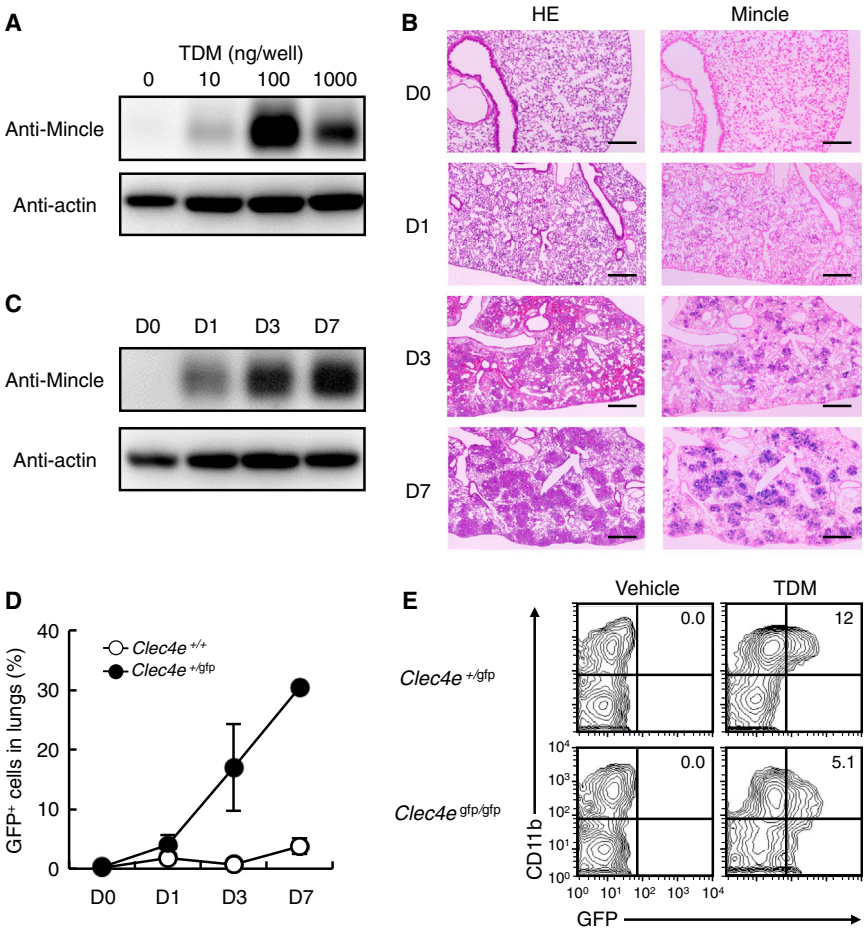

Figure 1. Mincle Is Induced by TDM in *Clec4e*$^{gfp/gfp}$ Mice

(A) Immunoblotting of Mincle in BMDCs. BMDCs were stimulated with plate-coated TDM for 12 hr. Lysates were blotted with Mincle antibody (6D6) and actin antibody.

(B) mRNA expression of Mincle in lungs. Mice were injected intravenously with oil-in-water emulsion of TDM. Serial sections of lungs were subjected to hematoxylin-eosin staining (HE) and in situ hybridization for Mincle mRNA (Mincle). Scale bars represent 0.5 mm.

(C) Immunoblotting of Mincle in lungs. Lysates of lungs from TDM-injected mice were blotted with Mincle antibody (6D6) and actin antibody.

(D) GFP induction in *Clec4e*$^{+/gfp}$ mice upon TDM stimulation. *Clec4e*$^{+/+}$ and *Clec4e*$^{+/gfp}$ mice were injected intravenously with TDM. GFP^{+} cells in lungs were analyzed by flow cytometry.

(E) GFP expression in *Clec4e*$^{gfp/gfp}$ mice. *Clec4e*$^{+/gfp}$ and *Clec4e*$^{gfp/gfp}$ mice were injected with TDM. At day 3, whole lung cells were stained with CD11b antibody and analyzed by flow cytometry. Numbers in quadrants indicate percentage of CD11b^{+}GFP^{+} cells.

(F) GFP expression in *Clec4e*$^{gfp/gfp}$ × *Fcer1g*$^{-/-}$ mice. *Clec4e*$^{+/gfp}$ × *Fcer1g*$^{-/-}$ and *Clec4e*$^{gfp/gfp}$ × *Fcer1g*$^{-/-}$ mice were injected with TDM.

(G) Mincle antibody 1B6 blocks GFP induction in *Clec4e*$^{gfp/gfp}$ mice. Mice were injected intravenously with 500 μg of Mincle antibody 1B6 at 30 min before TDM injection. Right, percentage of GFP^{+} cells. **$p < 0.01$.

Data are presented as mean ± SD (D, G) and are representative of two (B, C, E–G) or three (A, D) separate experiments. See also Figure S1.

(Ishikawa et al., 2009; Schoenen et al., 2010; Wells et al., 2008; Yamasaki et al., 2008, 2009). These recent observations raise the possibility that MCL may also function as a PRR for pathogens.

In this study, we report that MCL is an FcRγ-coupled activating receptor that recognizes mycobacterial cord factor. MCL is constitutively expressed in myeloid cells and drives inducible Mincle expression upon stimulation by cord factor. Moreover, cord factor failed to induce innate and acquired immunity in MCL-deficient mice.

RESULTS

Mincle Is Induced by TDM in the Absence of Mincle Protein

Mincle is an essential receptor for TDM (Ishikawa et al., 2009; Schoenen et al., 2010). TDM stimulation induced Mincle expression in bone-marrow-derived dendritic cells (BMDCs) (Figure 1A), thus suggesting that an autoamplification loop of Mincle may operate through Mincle-mediated signaling. Furthermore, in vivo injection of TDM dramatically induced Mincle expression in the lungs, which overlapped with the granuloma area (Figure 1B). The induction of Mincle protein was also evident by an immunoblot analysis (Figure 1C). However, Mincle expression was barely detected under resting (unstimulated) conditions (Figures 1A–1C), which prompted us to hypothesize that another TDM receptor, rather than Mincle itself, may promote the initial induction of Mincle.

In order to determine which receptor is responsible for this initial induction, we established green fluorescent protein (GFP) reporter mice that enabled us to monitor Mincle expression in the absence of Mincle protein by inserting a GFP gene into the

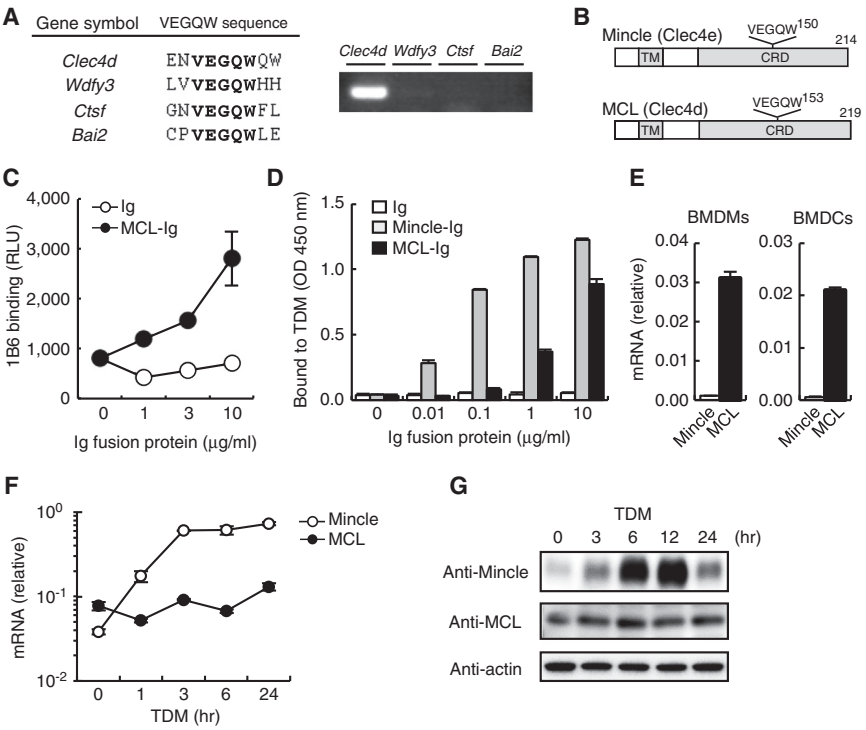

Figure 2. Identification of a C-type Lectin MCL as a TDM Receptor

(A) mRNA expression of genes containing 1B6-epitope sequence. Genes containing VEGQW sequence according to NCBI protein BLAST database except for Mincle. mRNA expression of these genes in lungs was detected by RT-PCR.

(B) Schematic representations of Mincle and MCL. TM, transmembrane.

(C) Binding of 1B6 to MCL. hIgG$_1$-Fc (Ig) and MCL-Ig were incubated with biotinylated 1B6. 1B6 binding to Ig-fusion protein was measured as luminescence induced by energy transfer as described in Experimental Procedures.

(D) MCL binds to TDM. Ig, Mincle-Ig, and MCL-Ig were incubated with 0.2 μg/well plate-coated TDM. Bound proteins were detected with anti-hIgG-HRP.

(E) mRNA expression of Mincle and MCL. RT-PCR was performed with mRNA from BMDMs and BMDCs.

(F and G) Expression of Mincle and MCL upon stimulation. BMDCs were stimulated with TDM for indicated periods and the expression amount of mRNA (F) and protein (G) were determined.

Data are presented as mean ± SD (C–F) and are representative of two (A, C, G) or three (D–F) separate experiments. See also Figure S2.

Mincle genomic locus (*Clec4e*$^{gfp/gfp}$) (Figure S1 available online). BMDCs from Mincle-sufficient heterozygous (*Clec4e*$^{+/gfp}$) mice showed induced expression of GFP as well as Mincle upon stimulation, suggesting that GFP from the *Clec4e*^{gfp} allele faithfully reflected Mincle expression (Figure S1D). *Clec4e*$^{gfp/gfp}$ BMDCs lost the expression of the Mincle protein, as expected. Meanwhile, TDM injection into *Clec4e*$^{+/gfp}$ mice increased GFP expression in pulmonary CD11b^{+} cells (Figures 1D and 1E, top). GFP was also induced by TDM even in Mincle-deficient *Clec4e*$^{gfp/gfp}$ mice, albeit to a lesser extent (Figure 1E, bottom). This implies that ligand-induced Mincle expression could be initiated by a receptor(s) other than Mincle. The GFP expression in *Clec4e*$^{gfp/gfp}$ mice was totally abolished when they were crossed with *Fcer1g*$^{-/-}$ mice, suggesting that FcRγ is required for the "Mincle-independent" receptor signaling (Figure 1F). GFP induction was inhibited by a Mincle antibody (1B6) even in the absence of Mincle (*Clec4e*$^{gfp/gfp}$) (Figure 1G, bottom). These results raised the possibility that a molecule that cross-reacts with the mAb (1B6) may act as a TDM receptor through FcRγ.

MCL Is a TDM Receptor

We have previously identified the epitope sequence of the Mincle antibody 1B6 as a valine-glutamic acid-glycine-glutamine-tryptophan (VEGQW) sequence within the carbohydrate recognition domain (CRD) of Mincle (Yamasaki et al., 2008). We therefore searched for any protein(s) carrying this sequence in silico. The alignment of the primary amino acid sequence on the murine protein database identified four individual proteins that contain a VEGQW sequence (Figure 2A, left). Among them, only Clec4d (also called MCL) possessed a transmembrane region and was also strongly expressed in the lungs (Figure 2A, right; Balch et al., 1998; Simonsen et al., 2004). MCL is a type II transmembrane protein that shares high homology with Mincle and possesses a VEGQW sequence within its CRD (Figures 2B and S2N). As expected, 1B6 cross-reacted with a MCL-Ig fusion protein as assessed by an in vitro binding assay (Figure 2C). We further examined whether MCL was capable of recognizing TDM. Soluble MCL protein could bind to plate-coated TDM and synthetic analog trehalose dibehenate (TDB), indicating that MCL recognized cord factor directly (Figures 2D, S2A, S2B, and S2K–S2M). These results suggest that MCL is a TDM receptor.

We then examined the expression of MCL in myeloid cells. A large amount of MCL, but not Mincle, was constitutively expressed in bone-marrow-derived macrophages (BMDMs) and BMDCs (Figures 2E, S2C, and S2D). The expression of MCL was not limited to bone-marrow-derived cells, as shown by the fact that freshly isolated DC subsets, tissue macrophages, and neutrophils but not T cells abundantly expressed MCL in the steady state (Figures S2E–S2J; Graham et al., 2012).

We next investigated the gene induction of MCL upon exposure to TDM stimuli. The expression of Mincle mRNA was dramatically induced in BMDCs, whereas MCL mRNA was expressed even in the resting state and was only slightly induced upon stimulation (Figure 2F). We also confirmed this at the protein expression level, because Mincle, but not MCL, was upregulated upon stimulation (Figure 2G). Therefore, the regulation of the gene expression of these two receptors differ substantially, i.e., MCL is constitutive, whereas Mincle is inducible.

MCL Is an FcRγ-Coupled Receptor

Mincle is known to be an FcRγ-coupled receptor (Yamasaki et al., 2008). We therefore investigated whether MCL was associated with FcRγ, although we initially thought this was unlikely, because MCL does not possess any of the charged residues that mediate the interaction with FcRγ in many other receptors (Robinson et al., 2006), including Mincle. However, MCL was

found to be expressed on the cell surface only in the presence of FcRγ, suggesting that MCL also forms a complex with FcRγ (Figure 3A). The binding seemed to be selective for FcRγ, because other signaling subunits, such as DAP10 or DAP12, did not have any impact on the surface expression of MCL (Figure 3A). Indeed, MCL was coimmunoprecipitated with the FcRγ chain in an ectopic expression system (Figure 3B). Importantly, the association between endogenous MCL and FcRγ was also evident in a DC line (data not shown) and BMDCs (Figure 3C). Taken together, the requirement for FcRγ demonstrated in Figure 1F may be explained by the fact that it is a subunit of MCL.

Instead of a positively charged residue within the transmembrane region, MCL uniquely possesses a hydrophilic threonine (T38) at the corresponding position of the charged arginine (R42) in Mincle. We therefore examined the contribution of T38 to the binding to FcRγ by introducing a mutation in this residue. Substitution of T38 with a hydrophobic amino acid (T38L) abolished the binding with FcRγ, whereas the replacement with a hydrophilic residue (T38S) preserved the binding capacity (Figure 3D). These results suggest that the hydrophilicity of this position may play a role in the binding to FcRγ. The hydrophilic residue (threonine or serine) at this location is found in other mammalian species.

MCL Is an Activating Receptor for TDM

We next analyzed whether MCL is capable of transducing signals through FcRγ. Flag-tagged MCL was introduced into reporter cells expressing FcRγ (Yamasaki et al., 2008). Upon stimulation by plate-coated Flag antibody, MCL activated a nuclear factor of activated T cells (NFAT)-GFP reporter as potently as did Mincle (Figure 3E). Thus, MCL functions as an FcRγ-coupled activating receptor.

We also stimulated these cells with TDM and found that TDM activated the NFAT reporter through MCL (Figure 3F). In line with the findings shown in Figure 2D, the ligand recognition of MCL seemed to be weaker than that of Mincle (Figure 3F). Importantly, human MCL also recognized TDM (Figure S3).

We further confirmed the reactivity of 1B6 mAb against MCL by using these transfectants. A Flag antibody was used to verify that Mincle-Flag and MCL-Flag were expressed at comparable intensities (Figure 3G, anti-Flag). MCL could indeed be recognized by the bispecific mAb 1B6, albeit with weaker potency compared to Mincle (Figure 3G, 1B6). In sharp contrast, each specific antibody recognized either Mincle or MCL alone (Figure 3G, bottom). The introduction of a mutation within the VEGQW sequence of MCL ($MCL^{\Delta VEGQW}$) abolished the binding of 1B6, indicating that 1B6 cross-reacted with MCL through an identical epitope that was present in Mincle (data not shown) (Yamasaki et al., 2008).

The 1B6 antibody blocked MCL-TDM binding even more potently than it did against Mincle-TDM interaction, probably because MCL bound to TDM weakly (Figure 3H). These results suggest that the blocking effect of 1B6 in Mincle-deficient mice demonstrated in Figure 1G was probably due to the neutralization of MCL.

Defective Innate Immunity in MCL-Deficient Mice

To gain a better understanding of the physiological significance of MCL, we established MCL-deficient mice (Figures S4A–S4C). *Clec4d*$^{-/-}$ mice were born according to the Mendelian law and showed no obvious abnormalities, and the cellularity of their thymus, spleen, lymph node, and lung were not altered (data not shown).

In order to compare the TDM responses in vitro, BMDCs and BMDMs were prepared from WT, *Clec4d*$^{-/-}$, *Clec4e*$^{-/-}$, and *Fcer1g*$^{-/-}$ mice. The TDM-induced cytokine production was totally dependent on Mincle and its signaling subunit, FcRγ, as previously reported (Figures 4A and 4B; Ishikawa et al., 2009; Schoenen et al., 2010). The production of these cytokines was still severely impaired in *Clec4d*$^{-/-}$ cells (Figures 4A and 4B).

We next examined the in vivo innate immune responses. A single injection of TDM into mice caused lethal systemic inflammation, whereas *Clec4d*$^{-/-}$ and *Clec4e*$^{-/-}$ mice were, respectively, partially and completely resistant (Figure 4C). TDM-induced thymic atrophy was also dependent on MCL (Figure S4D), as it is on Mincle (Ishikawa et al., 2009). TDM caused lung inflammation, including an increase in lung weight index and granuloma formation, which were significantly suppressed in *Clec4d*$^{-/-}$ mice (Figures 4D and 4E). This was also true for cytokine mRNA expression in the lungs (Figure 4F). Therefore, MCL is still required for TDM-mediated innate immunity, even though Mincle is essential for such immunity.

MCL Drives Mincle Expression

We next tried to determine why these two receptors are individually required for the innate immune responses. Two possible explanations are as follows: (1) Mincle and MCL may synergistically operate for downstream signaling or (2) some hierarchical relationship may exist between Mincle and MCL, e.g., MCL regulates Mincle expression. To address the first idea, Mincle and MCL were coexpressed in reporter cells to see whether these two receptors synergistically augment downstream signaling (Figure 5A). However, we detected no synergy between Mincle and MCL in this system (Figure 5B).

Given that MCL is constitutively expressed prior to the upregulation of Mincle (Figures 2E and 2G), MCL might play a role in the induction of Mincle in response to TDM, as suggested in Figure 1E. We therefore examined the Mincle upregulation in the presence and absence of MCL. The Mincle protein was induced upon TDM stimulation in WT cells, whereas it was severely suppressed in *Clec4d*$^{-/-}$ cells (Figure 5C). This was also true for the mRNA, as shown by the fact that Mincle induction was impaired in *Clec4d*$^{-/-}$ cells (Figure 5D, left). Slight induction of Mincle in *Clec4d*$^{-/-}$ mice was probably due to signaling through low amount of Mincle itself (Figure S5). In contrast, Mincle was dispensable for MCL expression (Figure 5D, right). Furthermore, Mincle induction upon TDM injection in vivo was also compromised in *Clec4d*$^{-/-}$ mice (Figure 5E).

Defective responses in *Clec4d*$^{-/-}$ DCs were restored by reconstituting the cells with exogenous Mincle (Figures 5F and 5G). Based on the above findings, we conclude that MCL is critically involved in TDM-mediated innate immune responses by driving Mincle expression.

MCL Promotes DC Maturation to Prime T Cells

We then asked whether Mincle and MCL contribute to T cell priming in vitro, because TDM has been known as a potent adjuvant (Numata et al., 1985; Sakurai et al., 1989; Werninghaus

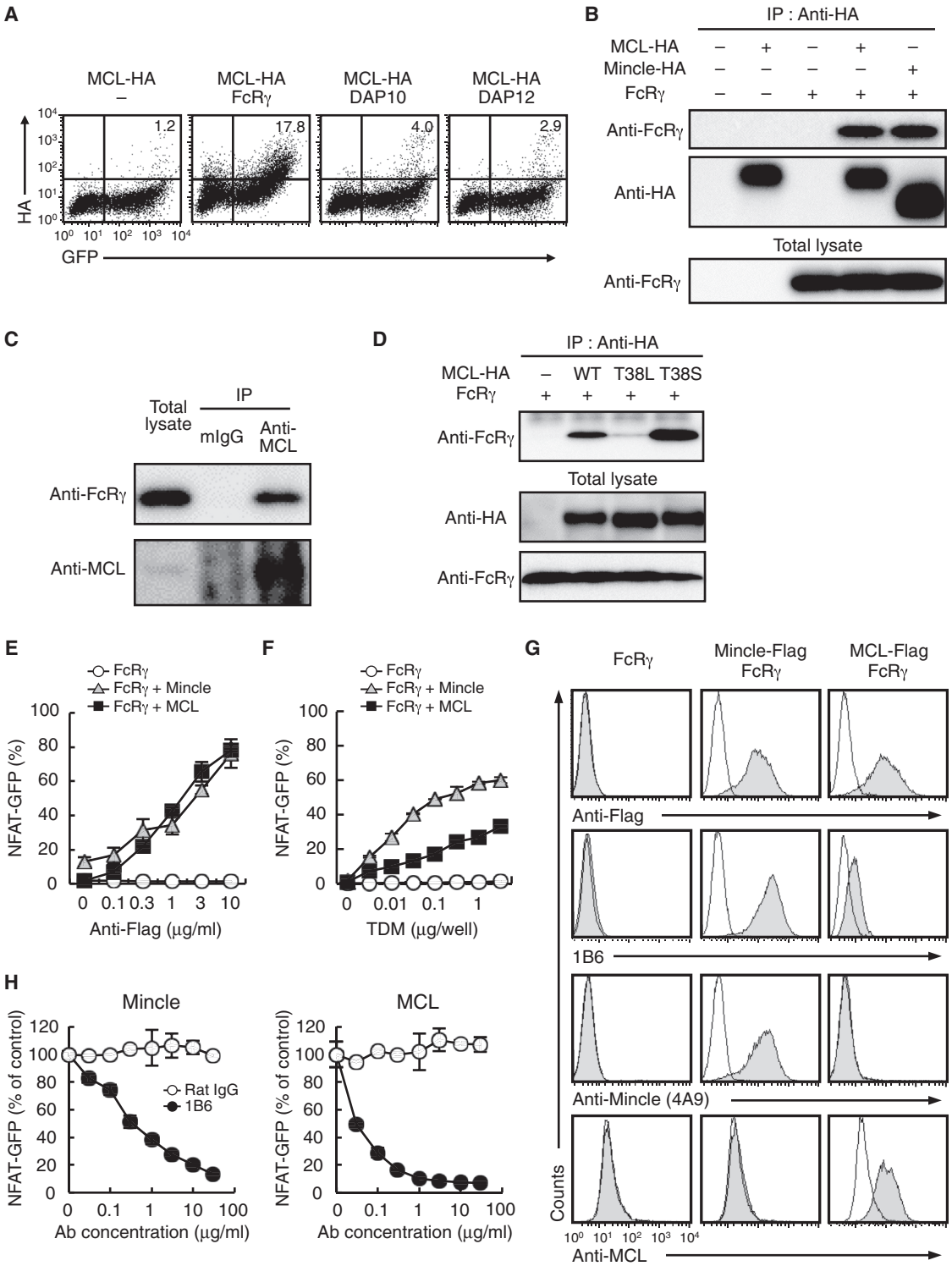

Figure 3. MCL Is an FcRγ-Coupled TDM Receptor

(A) FcRγ-dependent surface expression of MCL. HEK293T cells were transfected with HA-tagged MCL together with pMX-IRES-GFP vector alone (–), FcRγ, DAP10, or DAP12. Surface expression of MCL was detected by mAb against HA, which is attached at the extracellular region (C terminus) of MCL.

(B) MCL is associated with FcRγ. HEK293T cells were transfected with HA-tagged-MCL or -Mincle together with FcRγ. Lysates were immunoprecipitated with HA antibody and blotted with FcRγ antibody and HA antibody. Total lysates were also blotted with FcRγ antibody.

(C) Endogenous MCL is bound to FcRγ. Lysates from 1 × 10^7 cells of BMDCs were immunoprecipitated with mouse IgG or MCL antibody (66-1) and blotted with FcRγ antibody and MCL antibody (1K1-41).

(legend continued on next page)

et al., 2009). BMDCs were pulsed with the ovalbumin (OVA) antigen and cocultured with T cells from OVA-specific T cell receptor (TCR) transgenic (Tg) mice in the absence or presence of TDM. TDM enhanced the antigen-specific secretion of interferon-γ (IFN-γ) and interleukin-17 (IL-17) from $CD4^+$ OT-II T cells without affecting their proliferation (Figure 6A). However, the enhancement was severely suppressed in the absence of MCL, Mincle, and their common subunit FcRγ chain. Thus, MCL and Mincle can promote the development of T helper 1 (Th1) and Th17 cells (Schoenen et al., 2010).

This was also true for IFN-γ production from $CD8^+$ OT-I T cells (Figure 6B). In addition, both receptors were required for $CD8^+$ T cell activation when a whole antigen was presented on major histocompatibility complex (MHC) class I molecules through cross-presentation (Figure 6C). These results suggest that Mincle and MCL play a crucial role in T cell priming through DCs.

To investigate how the Mincle and MCL expressed on DCs support T cell priming, we examined the expression of costimulatory molecules on DCs. TDM induced the upregulation of CD80, CD86, and CD40 in WT DCs (Figure 6D). However, this induction was suppressed in *Clec4d*$^{-/-}$, *Clec4e*$^{-/-}$, and *Fcer1g*$^{-/-}$ DCs.

TDM upregulated chemokine receptor CCR7 on BMDCs, as assessed by CCL19-Fc binding, in a MCL- and Mincle-dependent manner (Figure 6D). Subcutaneous administration of TDM alone increased the number of $CD11c^+$ DCs in the draining lymph nodes, whereas this was compromised in *Clec4d*$^{-/-}$ and *Clec4e*$^{-/-}$ mice (Figure 6E). These results suggest that TDM induces the maturation and migration of DCs, and both Mincle and MCL contribute to these responses. MCL-mediated adjuvanticity was also verified by human MCL (Figure S6).

Defective Acquired Immunity in MCL-Deficient Mice

We then examined acquired immune responses in vivo. The immunization of mice with OVA antigen together with TDM greatly promoted footpad swelling, a typical delayed type hypersensitivity (DTH) response, upon secondary challenge with the antigen alone (Figure S7A). The TDM-induced DTH responses were significantly impaired in *Clec4e*$^{-/-}$ and *Clec4d*$^{-/-}$ mice (Figure 7A). This impairment was specific for TDM, as shown by the fact that footpad swelling elicited by the yeast adjuvant zymosan was not altered between the mice (Figure S7B). These results suggest that MCL and Mincle both mediate the adjuvant activity of TDM toward DTH responses. TDM augmented the production of OVA-specific IgG in WT mice during the same immunization protocol. However, this effect was impaired in *Clec4e*$^{-/-}$ mice and was partially reduced in *Clec4d*$^{-/-}$ mice (Figure 7B).

Finally, we performed a murine model of Th17-cell-mediated autoimmune disease, experimental autoimmune encephalomyelitis (EAE). WT mice were immunized with myelin oligodendrocyte glycoprotein (MOG) peptide together with various adjuvants. TDM, but not incomplete Freund's adjuvant (IFA) alone, was capable of eliciting EAE (Figure S7C). This was almost totally abrogated in *Clec4d*$^{-/-}$ mice, whereas only partial reduction was observed in *Clec4e*$^{-/-}$ mice (Figures 7C and 7D). Simultaneously, skin inflammation was observed at the injection sites of the same immunized WT mice (Martinez de la Torre et al., 2005). This pathology seemed to reflect an innate immune response, because it was induced by TDM even in *Rag1*$^{-/-}$ mice (Figure S7D). In sharp contrast to the EAE, the skin inflammation was completely abolished in *Clec4e*$^{-/-}$ but not in *Clec4d*$^{-/-}$ mice (Figure 7E). Thus, Mincle and MCL differentially contribute to innate inflammation and T cell-dependent EAE, respectively. Indeed, splenocytes from *Clec4e*$^{-/-}$ and *Clec4d*$^{-/-}$ mice had lower IL-17 expression in the recall responses against ex vivo stimulation with MOG peptide (Figure 7F).

These results suggest that MCL critically contributes to acquired immunity, particularly to EAE development. Mincle induction might be impaired in *Clec4d*$^{-/-}$ mice during acquired immunity, but the contribution of MCL to EAE was clearly Mincle independent (Figures 7C and 7D).

MCL and Mincle Contribute to Mycobacterial Infection

We finally examined the contribution of MCL and Mincle to mycobacterial infection in vitro. The induction of tumor necrosis factor (TNF) and MIP-2 mRNA upon *M. tuberculosis* H37Rv infection was significantly impaired in *Clec4d*$^{-/-}$ and *Clec4e*$^{-/-}$ BMDMs (Figure 7G). Mycobacteria-induced Mincle induction was also severely impaired in *Clec4d*$^{-/-}$ BMDMs (Figure 7H).

WT, *Clec4d*$^{-/-}$, and *Clec4e*$^{-/-}$ mice were infected with *Mycobacterium bovis* Bacille de Calmette et Guérin (BCG) and we examined host immune responses against mycobacteria. The IFN-γ production in response to PPD was partially impaired in the *Clec4d*$^{-/-}$ and *Clec4e*$^{-/-}$ mice (Figure 7I).

These results suggest that MCL and Mincle contribute to the immune responses against mycobacteria.

DISCUSSION

In this study, we showed that the C-type lectin MCL is an FcRγ-coupled activating receptor for a mycobacterial adjuvant, TDM. In support of this finding, a recent study reported that MCL functions as an activating receptor (Graham et al., 2012). The acquisition of different receptors with distinct functions, but that share the same ligand, would allow the host to more finely regulate the immune responses.

(D) Mutagenesis analysis of transmembrane region in MCL. HEK293T cells were transfected with HA-tagged MCL (WT) or mutant MCL (T38L and T38S) together with FcRγ. Lysates were immunoprecipitated with HA antibody and blotted with FcRγ antibody. Total lysates were also blotted with HA antibody and FcRγ antibody.

(E) MCL transduces activation signal through FcRγ. NFAT-GFP reporter cells were transfected with FcRγ together with Flag-tagged-Mincle or -MCL. Cells were stimulated with plate-coated Flag antibody for 24 hr. Induction of NFAT-GFP was analyzed by flow cytometry.

(F) TDM signals through MCL-FcRγ axis. Reporter cells were stimulated with plate-coated TDM for 24 hr.

(G) 1B6 recognizes MCL. 2B4 cells expressing FcRγ alone or together with Flag-tagged-Mincle or -MCL were stained with Flag antibody, Mincle antibody (1B6 and 4A9), and MCL antibody (1K2-5). Open histograms show staining with isotype control.

(H) 1B6 blocks TDM recognition by MCL. Mincle or MCL reporter cells were treated with 1B6 or rat IgG, followed by stimulation with TDM (1 μg/well).

Data are presented as mean ± SD (E, F, H) and are representative of two (C) or three (A, B, D–H) separate experiments. See also Figure S3.

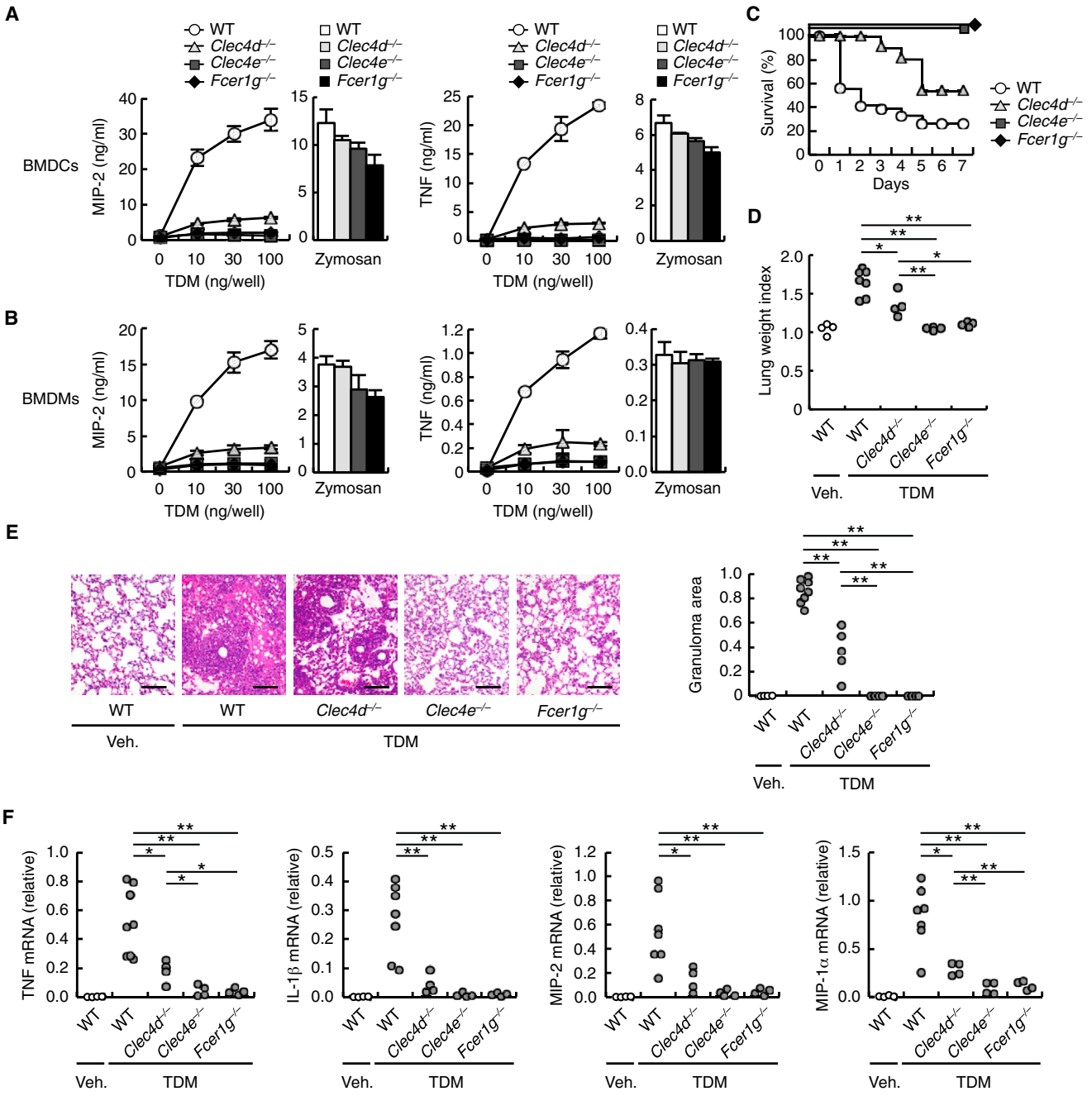

Figure 4. Impaired Innate Immune Responses in *Clec4d*$^{-/-}$ Mice

(A and B) TDM-induced cytokine production in vitro. BMDCs (A) and BMDMs (B) from WT, *Clec4d*$^{-/-}$, *Clec4e*$^{-/-}$, and *Fcer1g*$^{-/-}$ mice were stimulated with plate-coated TDM or zymosan (100 μg/ml) for 48 hr. Concentrations of MIP-2 and TNF were determined by ELISA. Data are presented as mean ± SD and are representative of three separate experiments.

(C) Lethal systemic inflammation by TDM. Survival curve of WT (n = 33), *Clec4d*$^{-/-}$ (n = 11), *Clec4e*$^{-/-}$ (n = 10), and *Fcer1g*$^{-/-}$ (n = 10) mice after intravenous administration of oil-in-water emulsion of TDM.

(D) TDM-induced lung swelling. At day 7 after injection of TDM or vehicle alone (veh.), lung swelling was evaluated by lung weight index (LWI).

(E) TDM-induced granuloma formation. Histology of lungs was examined by HE staining at day 7 after TDM injection. Scale bars represent 0.1 mm. Area of the granulomas was calculated as ratio of granuloma area/total lung area and shown in right.

(F) TDM-induced proinflammatory mediators. mRNA expression of TNF, IL-1β, MIP-2, and MIP-1α in the lungs at day 7 after TDM injection were evaluated by real-time PCR. *$p < 0.05$; **$p < 0.01$.

See also Figure S4.

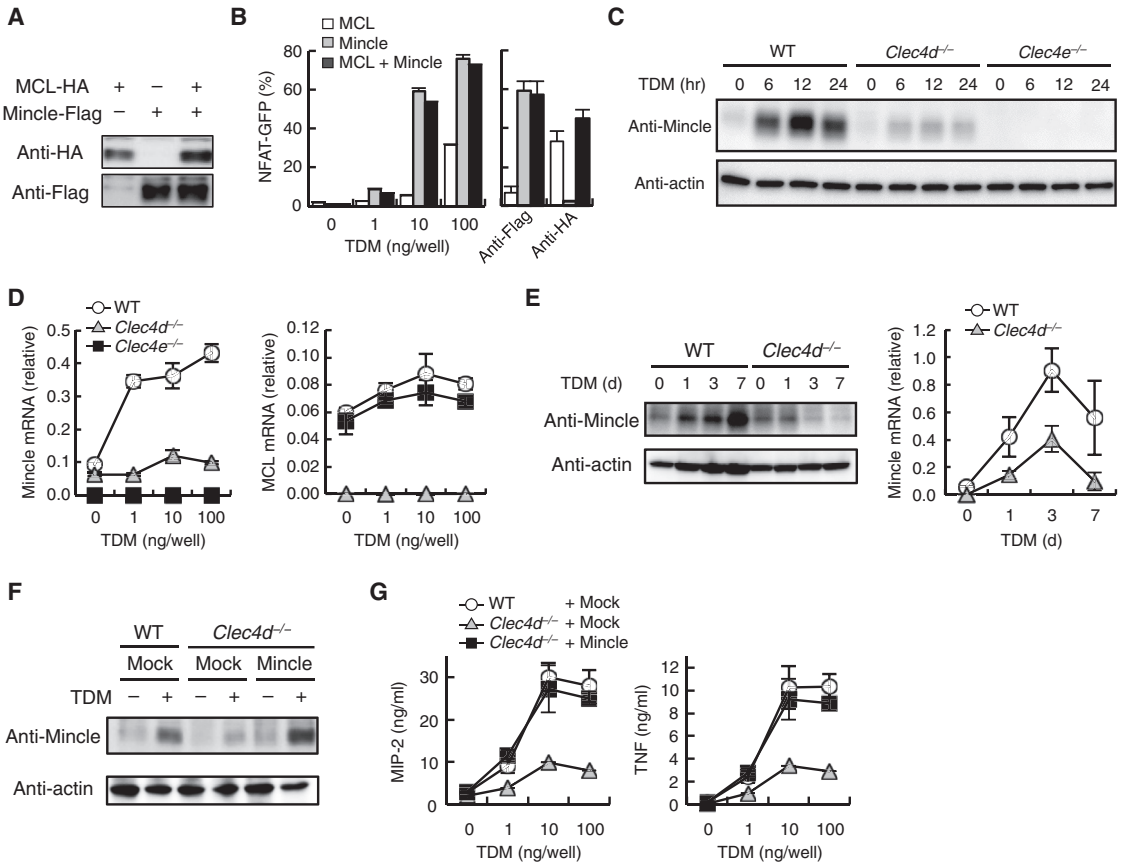

Figure 5. MCL Drives Inducible Expression of Mincle by TDM

(A and B) Coexpression of MCL and Mincle. Reporter cells were transfected with HA-tagged MCL and/or Flag-tagged Mincle (A). These cells were stimulated with TDM, HA antibody, and Flag antibody for 24 hr and GFP expression was determined (B).

(C) Immunoblotting of Mincle in BMDCs. BMDCs from WT, *Clec4d*$^{-/-}$, and *Clec4e*$^{-/-}$ mice were stimulated with TDM for indicated times. Lysates were blotted with Mincle antibody and actin antibody.

(D) mRNA expression of Mincle and MCL in BMDCs. BMDCs were stimulated with TDM for 6 hr. mRNA expression of Mincle and MCL was measured by RT-PCR.

(E) Mincle induction in lungs. WT and *Clec4d*$^{-/-}$ mice were injected with TDM and lungs were obtained at indicated days after injection. Immunoblotting of Mincle and actin (left). mRNA expression of Mincle was shown (right).

(F and G) Restoration of *Clec4d*$^{-/-}$ DC function by exogenous Mincle. BMDCs were transduced with Mincle through lentivirus vector, followed by stimulation with TDM. Lysates were blotted with Mincle antibody and actin antibody (F). Concentrations of MIP-2 and TNF were determined by ELISA (G).

Data are presented as mean ± SD (B, D, E, G) and are representative of two separate experiments. See also Figure S5.

The in vitro and in vivo innate immune responses induced by TDM were almost completely abrogated in Mincle-deficient mice (Ishikawa et al., 2009; Schoenen et al., 2010), indicating that MCL cannot substitute for Mincle. Therefore, we did not initially expect that MCL deficiency would influence the TDM-mediated innate immune responses. However, MCL was crucial for these responses, presumably by initiating ligand-mediated Mincle induction, as follows. (1) MCL is a dominant TDM receptor at the resting stage. (2) TDM binds to MCL to initiate Mincle expression, probably through C/EBPβ (Matsumoto et al., 1999), and (3) TDM binds to MCL and Mincle, which efficiently induce Mincle expression. (4) TDM binds to a sufficient amount of high-affinity receptor Mincle and delivers signals that might be strong enough to induce innate immune responses. MCL-intrinsic signaling capacity was also verified by anti-MCL-mediated crosslinking in WT cells.

Although the coexpression of Mincle and MCL did not show a synergistic effect, at least in the reporter cell line, some physical or functional interaction might be operating in the context of myeloid cells. The possibility of heterophilic oligomerization between Mincle and MCL cannot be excluded, as occurs with the NKG2-CD94 and Dectin-1-Galectin-3 dimers (Esteban et al., 2011; Sullivan et al., 2007). Therefore, the cooperation between Mincle and MCL is still an intriguing issue that needs to be clarified.

In contrast to innate immune responses, TDM-induced EAE was quite different, namely the symptom was only partially suppressed in *Clec4e*$^{-/-}$ mice, although MCL was indispensable for EAE. Thus, MCL is capable of mediating such acquired immunity independent of Mincle. The precise molecular mechanisms by which MCL predominantly contributes to EAE are currently unclear. Resistance to EAE in *Clec4d*$^{-/-}$ mice was not due to the mixed genetic background; a similar contrast was

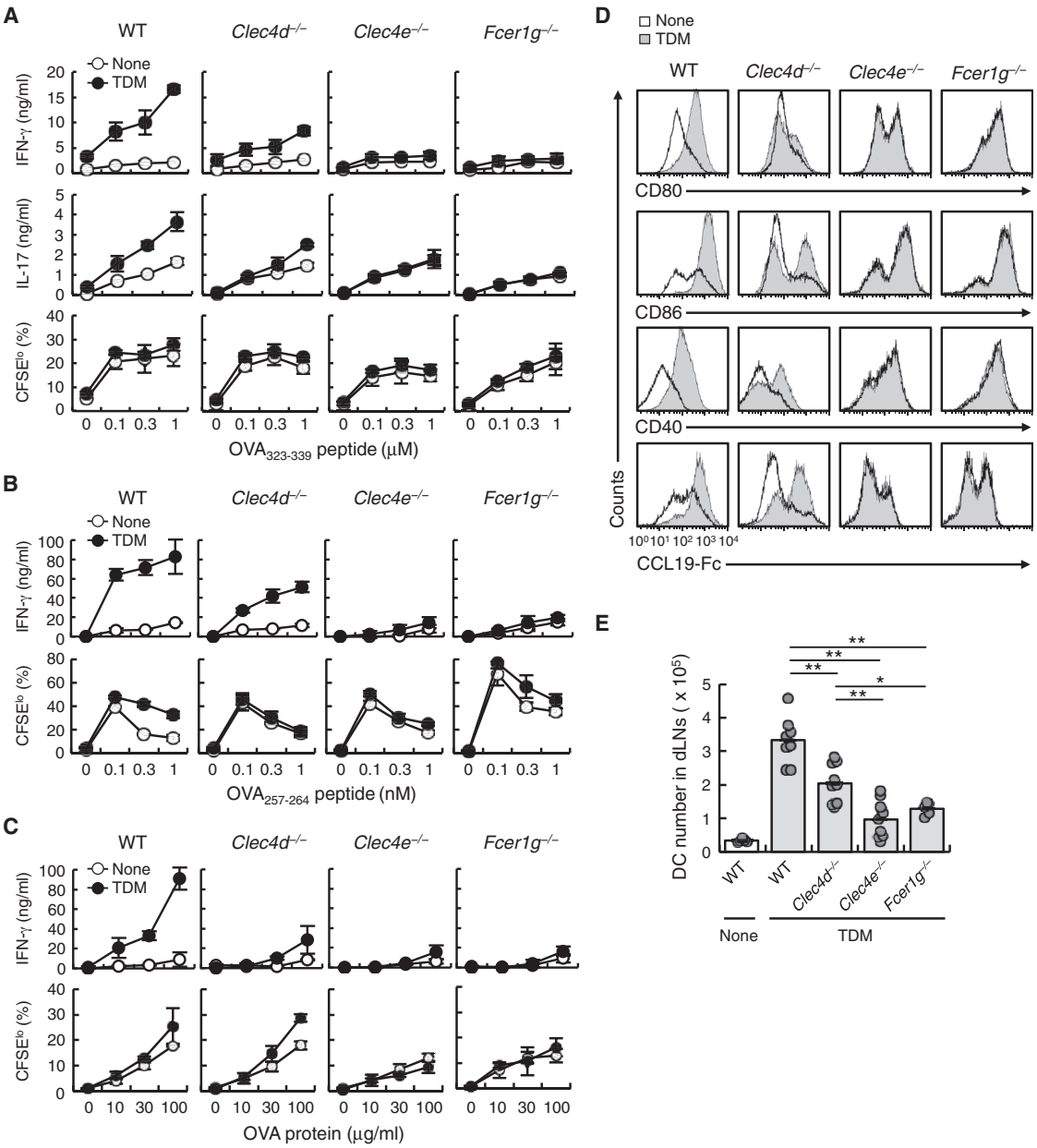

Figure 6. Impaired APC Functions in *Clec4d*$^{-/-}$ DCs

(A) TDM-enhanced CD4$^+$ T cell function. BMDCs from WT, *Clec4d*$^{-/-}$, *Clec4e*$^{-/-}$, and *Fcer1g*$^{-/-}$ mice were left untreated (open circles) or stimulated with plate-coated TDM (closed circles) and then cocultured with CFSE-labeled OT-II cells and $OVA_{323-339}$ peptide for 2 days. Concentrations of IFN-γ and IL-17 were measured by ELISA. Cell proliferation was evaluated by CFSE dilution.

(B and C) TDM-enhanced CD8$^+$ T cell function. TDM-stimulated BMDCs were cocultured with CFSE-labeled OT-I cells and $OVA_{257-264}$ peptide (B) or OVA protein (C) for 2 days.

(D) Upregulation of costimulatory molecules by TDM. BMDCs were left untreated or stimulated with TDM for 48 hr. Surface expression of CD80, CD86, CD40, and CCR7 was analyzed as indicated.

(E) TDM-induced DC accumulation in draining LNs. Mice were injected with oil-in-water emulsion of TDM at the base of the tail. Inguinal LNs were collected at 7 days after TDM injection. The number of CD11c$^+$ cells was analyzed by flow cytometry. *$p < 0.05$, **$p < 0.01$.

Data are presented as mean ± SD (A–C) and are representative of three (A–D) separate experiments. See also Figure S6.

also evident when *Clec4d*$^{-/-}$ mice were compared with littermate *Clec4d*$^{+/+}$ mice (data not shown). Thus far, we have not found any MCL-specific APC functions, at least in BMDCs. MCL, but not Mincle, is reported to be an endocytotic receptor (Arce et al., 2004), so it is possible that MCL may uniquely promote antigen processing or trafficking in some particular DC subsets, as was reported for Clec9a (Sancho et al., 2009). Alternatively, it is tempting to speculate that γδ T cells expressing MCL and FcRγ

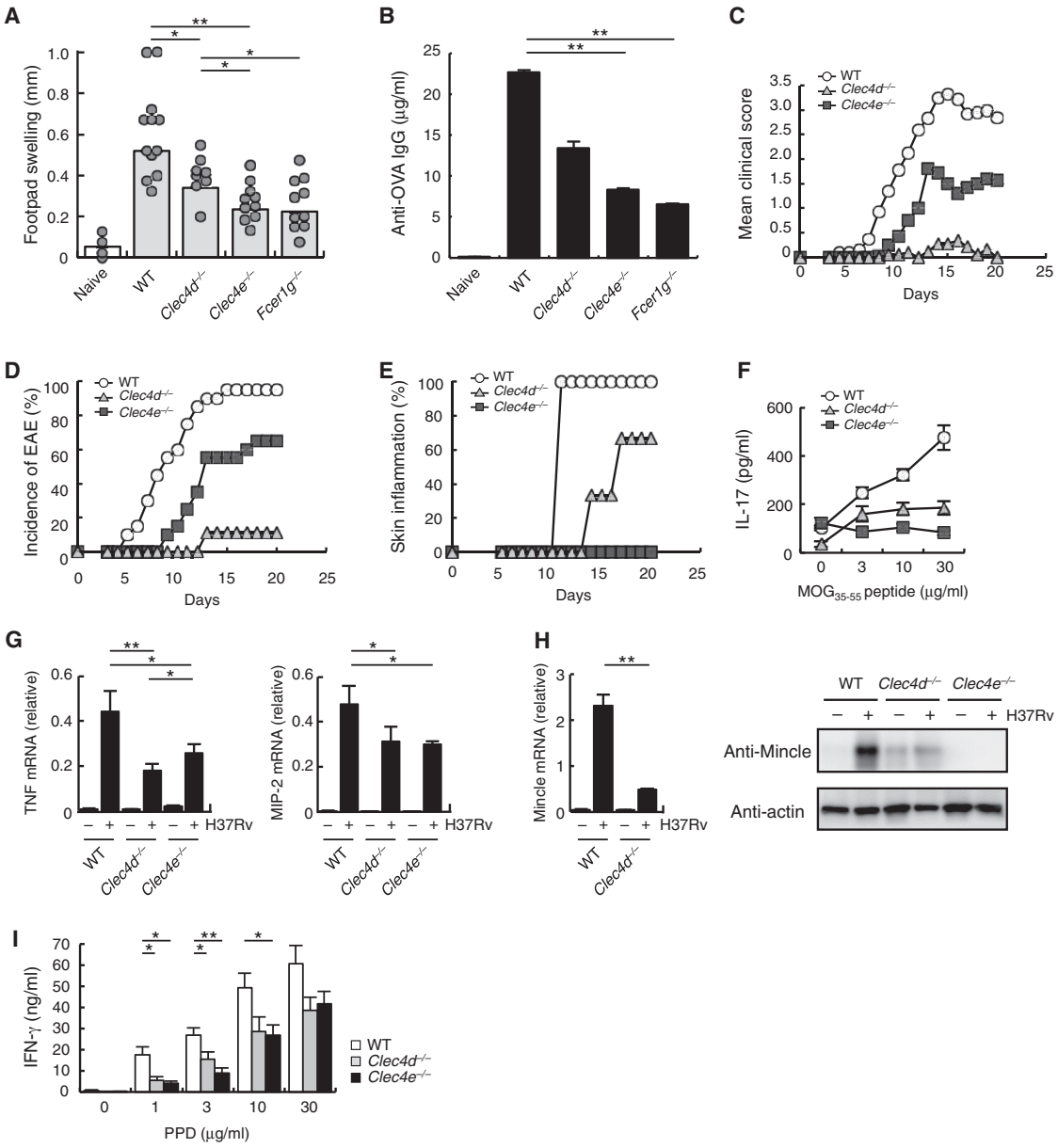

Figure 7. Critical Role of MCL in Acquired Immune Responses

(A) TDM-induced DTH response. Mice were immunized with OVA in oil-in-water emulsion of TDM, followed by injection of OVA into footpads at 7 days after immunization. Footpad thickness was measured at 24 hr after challenge. Each symbol represents an individual mouse.

(B) Antigen-specific antibody production. Serum was collected at 5 days after OVA challenge as described in (A). Concentration of anti-OVA IgG was determined by ELISA. Naive (n = 7), WT (n = 12), *Clec4d*$^{-/-}$ (n = 8), *Clec4e*$^{-/-}$ (n = 10), and *Fcer1g*$^{-/-}$ (n = 10) mice were used in this experiment.

(C and D) TDM-induced EAE. WT (n = 20), *Clec4d*$^{-/-}$ (n = 9), and *Clec4e*$^{-/-}$ (n = 20) mice were immunized with MOG_{35-55} peptide in IFA containing TDM. The disease severity of each mouse was scored, and the mean clinical score (C) and disease incidence (D) at the indicated times were plotted.

(E) TDM-induced skin inflammation. The incidence of skin inflammation at the immunization site of EAE-treated mice was shown. See also Figure S7D.

(F) Recall response against MOG antigen. Mice were rechallenged with MOG_{35-55} peptide and pertussis toxin at 20 days after immunization for EAE. Spleens were collected at 4 days after rechallenge and stimulated with MOG_{35-55} peptide for 96 hr. Concentrations of IL-17 were determined by ELISA. Data are representative of two separate experiments. WT (n = 7), *Clec4d*$^{-/-}$ (n = 7), and *Clec4e*$^{-/-}$ (n = 7) mice were used in total.

(G and H) Mycobacterial infection in vitro. BMDMs were infected with *M. tuberculosis* H37Rv, and mRNA expressions of TNF, MIP-2 (G), and Mincle (H, left) 24 hr after infection were determined by RT-PCR. Immunoblotting of Mincle and actin is shown (H, right).

(I) WT, *Clec4d*$^{-/-}$, and *Clec4e*$^{-/-}$ mice (n = 5 for each) were intratracheally infected with *M. bovis* BCG. Spleens were obtained 3 weeks after infection and stimulated with PPD for 4 days and concentration of IFN-γ in the supernatants was determined by ELISA for individual mice.

Data are presented as mean ± SEM (B, F, I) or SD (G, H). *p < 0.05; **p < 0.01. See also Figure S7.

may produce IL-17 in response to TDM in a TCR-independent manner (Martin et al., 2009). One might argue that MCL may contribute to the fundamental process of EAE development, rather than as a TDM receptor. However, this is unlikely because the *Clec4d*$^{-/-}$ mice developed EAE when we used an adjuvant that contains TLR ligands (data not shown).

The important question is what determines the functional difference between Mincle and MCL. Interestingly, the recognition of TDM by MCL appeared weaker than that by Mincle. Mincle possesses a conserved glutamic acid-proline-asparagine (EPN) sequence within its CRD (Drickamer, 1992). This EPN motif is critical for TDM recognition by Mincle (Ishikawa et al., 2009), but this motif was not conserved in MCL. These results imply that the low ligand affinity may be advantageous for MCL, presumably to limit the robustness of innate immune responses. On the contrary, constitutive MCL may deliver sustained signals, which may compensate for the signal intensity required for acquired immunity. Extensive comparisons of the affinity, stoichiometry, and signaling kinetics between Mincle and MCL may clarify a differential role for the two CLRs. Indeed, we have previously reported that the quantity and duration of FcRγ signals could lead to distinct immune responses (Yamasaki et al., 2004).

Alternatively, the functional difference between the two receptors may be solely attributable to their distributions or expression modes such as inducible versus constitutive. To dissect which of the "structural characteristics" or "expression patterns" determines the distinct function of these two CLRs, it would be intriguing to establish genetically targeted mice expressing Mincle instead of MCL, and vice versa.

Currently, the structural basis for the interaction of MCL to FcRγ through the hydrophilic residue remains unclear. This interaction may vary depending on the cell context (Graham et al., 2012). Recently, several receptors were reported to be associated with FcRγ or DAP12 despite their lack of a positively charged residue within their transmembrane region (Enomoto et al., 2010; Wines et al., 2006). Accumulating evidence of various modes of association with ITAM-bearing adaptors may expand the pool of candidate for potential ITAM-coupled receptors.

Mincle and some other C-type lectins recognize damage-associated molecular patterns (DAMPs) (Ahrens et al., 2012; Cambi and Figdor, 2009; Oka et al., 1998; Sancho et al., 2009; Yamasaki et al., 2008; Zhang et al., 2012). MCL might also function as a receptor for endogenous ligands, although we have not observed any evidence of this so far. The CLR family has a large number of members, and each of them has broad ligand specificity compared with that of TLRs, NLRs, and RLRs. These characteristics of CLRs may enable the host to recognize a wide variety of molecular patterns associated with pathogens and damaged self.

Recent developments in genomic sequencing of many species have shown that Mincle and MCL are not only homologous to each other, but are also highly conserved between species. Genomic annotation of the Mincle loci of various mammalian species revealed that monotremes and marsupialia possess only Mincle, but not MCL. However, placental mammals possess both Mincle and MCL, and they are located next to each other in the genome, thus suggesting that MCL may arise from the gene duplication of Mincle. Marsupialia have immature acquired immunity, particularly in neonates, compared with placentalia (Edwards et al., 2012). It is therefore speculated that MCL may have evolved along with the development of acquired immunity. The significance of MCL is verified by the fact that this gene is conserved among placental mammals.

It can be speculated that Mincle and MCL cooperatively function during the host defense against mycobacterial infection through distinct immune responses. Given that high mycobacterial loads in Mincle-deficient mice have recently been reported (Behler et al., 2012; Lee et al., 2012), a deficiency in antimycobacterial responses might be observed to be even more severe in Mincle-MCL double-deficient mice. Because the genes encoding Mincle and MCL are located within 30 kilobases of each other on mouse chromosome 6, double-deficient mice have to be generated by sequential targeting of these two genes in the same ES cell line, which is now under development.

It was reported that cationic liposome formulated with synthetic cord factor TDB, CAF01, has been developed as an adjuvant that potentially primes T cell responses in mice and humans (Aagaard et al., 2011; Agger et al., 2008; Christensen et al., 2009; Lindenstrøm et al., 2009). We confirmed that MCL, as well as Mincle, recognizes synthetic TDB (Ishikawa et al., 2009; Schoenen et al., 2010), suggesting that MCL may also be involved in mediating the adjuvant effect of the CAF01. Importantly, human MCL is also capable of activating acquired immune responses.

Although TDM is known as a potent adjuvant, some adverse effects, such as local inflammation and granulomagenesis, have been reported (Bekierkunst, 1968; Fomsgaard et al., 2011). It has been demonstrated that a synthetic TDM analog, 6,6′-Dideoxy-6,6′-bis-mycolylamino-α,α-trehalose (TDNM) or trehalose 6,6′-dicorynomycolate (TDCM) are less toxic, although they still potently activated antitumor activity (Sakurai et al., 1989; Watanabe et al., 1999). These observations may now be explained by the binding specificity of these analogs toward the two receptors. Less toxic adjuvants, such as TDNM or TDCM, may preferentially bind to MCL. It would be still possible that MCL may also recognize some unknown ligands in other pathogens.

Our identification of two TDM receptors with potentially distinct functions will provide information for the development of innovative adjuvants for vaccines. For instance, a synthetic ligand specific for MCL rather than Mincle may selectively enhance the cellular immunity while inducing minimal adverse effects derived from innate immune responses, which are largely dependent on Mincle (Ishikawa et al., 2009).

EXPERIMENTAL PROCEDURES

Mice

Clec4e$^{-/-}$ mice were generated previously (Yamasaki et al., 2009) and backcrossed at least eight generations with C57BL/6 mice. *Fcer1g*$^{-/-}$ mice of C57BL/6 genetic background were provided by T. Saito (RIKEN, Japan) (Park et al., 1998). *Clec4d*$^{-/-}$ and *Clec4e*$^{gfp/gfp}$ mice were established with E14.1 (129P2) embryonic stem cells and used as C57BL/6 and 129 mixed genetic background. C57BL/6 mice were obtained from Japan Clea. All mice were maintained in a filtrated-air laminar-flow enclosure and given standard laboratory food and water ad libitum. Animal protocols were approved by the committee of Ethics on Animal Experiment, Faculty of Medical Sciences, Kyushu University.

Homogeneous Proximity Assays

The binding of Ig-fusion proteins and 1B6 was determined by amplified luminescent proximity homogeneous assay according to the manufacturer's

recommendation (AlphaLISA, PerkinElmer). In brief, Ig-fusion proteins and biotinylated 1B6 (3 μg/ml) were incubated with anti-hIgG acceptor beads and streptavidin-coated donor beads. Oxygen-based energy transfer was detected by light production at 615 nm (EnSpire, PerkinElmer) and presented as relative light units (RLU).

Ig-Fusion Proteins

MCL-Ig and Mincle-Ig fusion proteins were prepared as described previously (Yamasaki et al., 2008). In brief, the C terminus of the extracellular domain of murine MCL (77–219 aa) or murine Mincle (46–214 aa) was fused to the N terminus of hIgG1 Fc region (mMCL-Ig and mMincle-Ig, respectively). The C terminus of the extracellular domain of human MCL (42–215 aa) or human Mincle (46–219 aa) was fused to the N terminus of hIgG1 Fc region (hMCL-Ig and hMincle-Ig, respectively). Ig-fusion proteins were incubated with 0.2 μg/well of plate-coated TDM, and bound proteins were detected by using HRP-labeled anti-human IgG. CCL19-Fc (14-1972-63) was purchased from eBioscience.

Granuloma Formation

TDM was prepared as oil-in-water emulsion consisting of mineral oil (9%), Tween-80 (1%), and PBS (90%) as previously described (Numata et al., 1985). 150 μl of emulsion containing 150 μg of TDM was injected intravenously into mice. Lungs were removed at 7 days after TDM injection and fixed in 10% formaldehyde for hematoxylin-eosin staining. Lung weight index was calculated as previously described (Ishikawa et al., 2009). A part of lungs were frozen for RNA analysis. The area of granuloma was quantified by ImageJ software.

Experimental Autoimmune Encephalomyelitis

Female mice were immunized by subcutaneous administration with 200 μg of $MOG_{35–55}$ peptide (Invitrogen) emulsified in IFA (Difco) containing 500 μg of TDM on day 0, supplemented with intravenous injection of 200 ng of pertussis toxin (List Biological Laboratories). Mice received an additional intraperitoneal injection of 200 ng pertussis toxin on day 2. Disease severity was scored as described previously (Miyake et al., 2007). For in vitro restimulation analysis, mice were injected intraperitoneally with 50 μg of $MOG_{35–55}$ peptide and 200 ng of pertussis toxin on day 20. Spleens were collected on day 24 and stimulated with $MOG_{35–55}$ peptide for 4 days. Concentrations of IL-17 in culture supernatants were determined by ELISA.

Delayed-type Hypersensitivity

Mice were sensitized by subcutaneous injection of 200 μg of OVA in oil-in-water emulsion of TDM (100 μg). At 7 days after sensitization, mice were challenged with 200 μg of heat-aggregated OVA (70°C, 1 hr) in 20 μl of PBS into both footpads. Footpad swelling was measured with vernier caliper and calculated as (footpad thickness after challenge) − (footpad thickness before challenge). Serum anti-OVA IgG was determined at day 5 after challenge by ELISA with anti-OVA IgG1 (2D11, Abcam) as a standard.

Mycobacterial Infection

For in vitro infection model, BMDMs were infected with *M. tuberculosis* H37Rv at a multiplicity of infection (MOI) of 1.0, and mRNA or lysates were prepared 24 hr after infection. For the in vivo infection model, the mice were infected intratracheally with 2×10^5 CFU of *M. bovis* BCG. Three weeks after infection, single-cell suspensions of splenocytes (5×10^5 cells) were stimulated with PPD (Japan BCG Laboratory) for 4 days, and the concentration of IFN-γ in culture supernatants was determined by ELISA.

Statistics

An unpaired two-tailed Student's t test was used for all the statistical analyses.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2013.03.010.

ACKNOWLEDGMENTS

We thank S. Kubo for generating mutant mice; Y. Fukui, E. Ishikawa, M. Nagata, S. Saijo, S. Maenaka, and M. Nakao for discussion; M. Tanaka for cDNA; H. Miyoshi for lentivirus vectors; M. Kurata, M. Shiokawa, Y. Sakakibara, and A. Nakayama for technical assistance; Y. Sanui-Nishi for secretarial assistance; and Laboratory, Medical Institute of Bioregulation, Kyushu University for technical support. This work was supported by Grant-in-Aid for Young Scientists (S), Funding Program for Next Generation World-Leading Researchers (NEXT Program), Takeda Science Foundation, the Uehara memorial foundation (S.Y.), Grant-in-Aid for Young Scientists (B), the Uehara Memorial foundation (Y.M.), and the Grant for Joint Research Project of the Institute of Medical Science, the University of Tokyo.

Received: September 14, 2012
Accepted: March 27, 2013
Published: April 18, 2013

REFERENCES

Aagaard, C., Hoang, T., Dietrich, J., Cardona, P.J., Izzo, A., Dolganov, G., Schoolnik, G.K., Cassidy, J.P., Billeskov, R., and Andersen, P. (2011). A multistage tuberculosis vaccine that confers efficient protection before and after exposure. Nat. Med. *17*, 189–194.

Agger, E.M., Rosenkrands, I., Hansen, J., Brahimi, K., Vandahl, B.S., Aagaard, C., Werninghaus, K., Kirschning, C., Lang, R., Christensen, D., et al. (2008). Cationic liposomes formulated with synthetic mycobacterial cordfactor (CAF01): a versatile adjuvant for vaccines with different immunological requirements. PLoS ONE *3*, e3116.

Ahrens, S., Zelenay, S., Sancho, D., Hanč, P., Kjær, S., Feest, C., Fletcher, G., Durkin, C., Postigo, A., Skehel, M., et al. (2012). F-actin is an evolutionarily conserved damage-associated molecular pattern recognized by DNGR-1, a receptor for dead cells. Immunity *36*, 635–645.

Arce, I., Martínez-Muñoz, L., Roda-Navarro, P., and Fernández-Ruiz, E. (2004). The human C-type lectin CLECSF8 is a novel monocyte/macrophage endocytic receptor. Eur. J. Immunol. *34*, 210–220.

Balch, S.G., McKnight, A.J., Seldin, M.F., and Gordon, S. (1998). Cloning of a novel C-type lectin expressed by murine macrophages. J. Biol. Chem. *273*, 18656–18664.

Behler, F., Steinwede, K., Balboa, L., Ueberberg, B., Maus, R., Kirchhof, G., Yamasaki, S., Welte, T., and Maus, U.A. (2012). Role of Mincle in alveolar macrophage-dependent innate immunity against mycobacterial infections in mice. J. Immunol. *189*, 3121–3129.

Bekierkunst, A. (1968). Acute granulomatous response produced in mice by trehalose-6,6-dimycolate. J. Bacteriol. *96*, 958–961.

Bloch, H. (1950). Studies on the virulence of tubercle bacilli; isolation and biological properties of a constituent of virulent organisms. J. Exp. Med. *91*, 197–218.

Cambi, A., and Figdor, C. (2009). Necrosis: C-type lectins sense cell death. Curr. Biol. *19*, R375–R378.

Christensen, D., Agger, E.M., Andreasen, L.V., Kirby, D., Andersen, P., and Perrie, Y. (2009). Liposome-based cationic adjuvant formulations (CAF): past, present, and future. J. Liposome Res. *19*, 2–11.

Drickamer, K. (1992). Engineering galactose-binding activity into a C-type mannose-binding protein. Nature *360*, 183–186.

Edwards, M.J., Hinds, L.A., Deane, E.M., and Deakin, J.E. (2012). A review of complementary mechanisms which protect the developing marsupial pouch young. Dev. Comp. Immunol. *37*, 213–220.

Enomoto, Y., Yamanishi, Y., Izawa, K., Kaitani, A., Takahashi, M., Maehara, A., Oki, T., Takamatsu, R., Kajikawa, M., Takai, T., et al. (2010). Characterization of leukocyte mono-immunoglobulin-like receptor 7 (LMIR7)/CLM-3 as an activating receptor: its similarities to and differences from LMIR4/CLM-5. J. Biol. Chem. *285*, 35274–35283.

Esteban, A., Popp, M.W., Vyas, V.K., Strijbis, K., Ploegh, H.L., and Fink, G.R. (2011). Fungal recognition is mediated by the association of dectin-1 and galectin-3 in macrophages. Proc. Natl. Acad. Sci. USA *108*, 14270–14275.

Flornes, L.M., Bryceson, Y.T., Spurkland, A., Lorentzen, J.C., Dissen, E., and Fossum, S. (2004). Identification of lectin-like receptors expressed by antigen presenting cells and neutrophils and their mapping to a novel gene complex. Immunogenetics *56*, 506–517.

Fomsgaard, A., Karlsson, I., Gram, G., Schou, C., Tang, S., Bang, P., Kromann, I., Andersen, P., and Andreasen, L.V. (2011). Development and preclinical safety evaluation of a new therapeutic HIV-1 vaccine based on 18 T-cell minimal epitope peptides applying a novel cationic adjuvant CAF01. Vaccine *29*, 7067–7074.

Graham, L.M., Gupta, V., Schafer, G., Reid, D.M., Kimberg, M., Dennehy, K.M., Hornsell, W.G., Guler, R., Campanero-Rhodes, M.A., Palma, A.S., et al. (2012). The C-type lectin receptor CLECSF8 (CLEC4D) is expressed by myeloid cells and triggers cellular activation through Syk kinase. J. Biol. Chem. *287*, 25964–25974.

Hardison, S.E., and Brown, G.D. (2012). C-type lectin receptors orchestrate antifungal immunity. Nat. Immunol. *13*, 817–822.

Ishikawa, E., Ishikawa, T., Morita, Y.S., Toyonaga, K., Yamada, H., Takeuchi, O., Kinoshita, T., Akira, S., Yoshikai, Y., and Yamasaki, S. (2009). Direct recognition of the mycobacterial glycolipid, trehalose dimycolate, by C-type lectin Mincle. J. Exp. Med. *206*, 2879–2888.

Lee, W.B., Kang, J.S., Yan, J.J., Lee, M.S., Jeon, B.Y., Cho, S.N., and Kim, Y.J. (2012). Neutrophils promote mycobacterial trehalose dimycolate-induced lung inflammation via the Mincle pathway. PLoS Pathog. *8*, e1002614.

Lindenstrøm, T., Agger, E.M., Korsholm, K.S., Darrah, P.A., Aagaard, C., Seder, R.A., Rosenkrands, I., and Andersen, P. (2009). Tuberculosis subunit vaccination provides long-term protective immunity characterized by multifunctional CD4 memory T cells. J. Immunol. *182*, 8047–8055.

Martin, B., Hirota, K., Cua, D.J., Stockinger, B., and Veldhoen, M. (2009). Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals. Immunity *31*, 321–330.

Martinez de la Torre, Y., Locati, M., Buracchi, C., Dupor, J., Cook, D.N., Bonecchi, R., Nebuloni, M., Rukavina, D., Vago, L., Vecchi, A., et al. (2005). Increased inflammation in mice deficient for the chemokine decoy receptor D6. Eur. J. Immunol. *35*, 1342–1346.

Matsumoto, M., Tanaka, T., Kaisho, T., Sanjo, H., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., and Akira, S. (1999). A novel LPS-inducible C-type lectin is a transcriptional target of NF-IL6 in macrophages. J. Immunol. *163*, 5039–5048.

Miyake, Y., Asano, K., Kaise, H., Uemura, M., Nakayama, M., and Tanaka, M. (2007). Critical role of macrophages in the marginal zone in the suppression of immune responses to apoptotic cell-associated antigens. J. Clin. Invest. *117*, 2268–2278.

Noll, H., Bloch, H., Asselineau, J., and Lederer, E. (1956). The chemical structure of the cord factor of *Mycobacterium tuberculosis*. Biochim. Biophys. Acta *20*, 299–309.

Numata, F., Nishimura, K., Ishida, H., Ukei, S., Tone, Y., Ishihara, C., Saiki, I., Sekikawa, I., and Azuma, I. (1985). Lethal and adjuvant activities of cord factor (trehalose-6,6′-dimycolate) and synthetic analogs in mice. Chem. Pharm. Bull. (Tokyo) *33*, 4544–4555.

Oka, K., Sawamura, T., Kikuta, K., Itokawa, S., Kume, N., Kita, T., and Masaki, T. (1998). Lectin-like oxidized low-density lipoprotein receptor 1 mediates phagocytosis of aged/apoptotic cells in endothelial cells. Proc. Natl. Acad. Sci. USA *95*, 9535–9540.

Park, S.Y., Ueda, S., Ohno, H., Hamano, Y., Tanaka, M., Shiratori, T., Yamazaki, T., Arase, H., Arase, N., Karasawa, A., et al. (1998). Resistance of Fc receptor-deficient mice to fatal glomerulonephritis. J. Clin. Invest. *102*, 1229–1238.

Robinson, M.J., Sancho, D., Slack, E.C., LeibundGut-Landmann, S., and Reis e Sousa, C. (2006). Myeloid C-type lectins in innate immunity. Nat. Immunol. *7*, 1258–1265.

Rogers, N.C., Slack, E.C., Edwards, A.D., Nolte, M.A., Schulz, O., Schweighoffer, E., Williams, D.L., Gordon, S., Tybulewicz, V.L., Brown, G.D., and Reis e Sousa, C. (2005). Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins. Immunity *22*, 507–517.

Saijo, S., Ikeda, S., Yamabe, K., Kakuta, S., Ishigame, H., Akitsu, A., Fujikado, N., Kusaka, T., Kubo, S., Chung, S.H., et al. (2010). Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against *Candida albicans*. Immunity *32*, 681–691.

Sakurai, T., Saiki, I., Ishida, H., Takeda, K., and Azuma, I. (1989). Lethal toxicity and adjuvant activities of synthetic TDM and its related compounds in mice. Vaccine *7*, 269–274.

Sancho, D., Joffre, O.P., Keller, A.M., Rogers, N.C., Martínez, D., Hernanz-Falcón, P., Rosewell, I., and Reis e Sousa, C. (2009). Identification of a dendritic cell receptor that couples sensing of necrosis to immunity. Nature *458*, 899–903.

Sato, K., Yang, X.L., Yudate, T., Chung, J.S., Wu, J., Luby-Phelps, K., Kimberly, R.P., Underhill, D., Cruz, P.D., Jr., and Ariizumi, K. (2006). Dectin-2 is a pattern recognition receptor for fungi that couples with the Fc receptor gamma chain to induce innate immune responses. J. Biol. Chem. *281*, 38854–38866.

Schoenen, H., Bodendorfer, B., Hitchens, K., Manzanero, S., Werninghaus, K., Nimmerjahn, F., Agger, E.M., Stenger, S., Andersen, P., Ruland, J., et al. (2010). Cutting edge: Mincle is essential for recognition and adjuvanticity of the mycobacterial cord factor and its synthetic analog trehalose-dibehenate. J. Immunol. *184*, 2756–2760.

Simonsen, A., Birkeland, H.C., Gillooly, D.J., Mizushima, N., Kuma, A., Yoshimori, T., Slagsvold, T., Brech, A., and Stenmark, H. (2004). Alfy, a novel FYVE-domain-containing protein associated with protein granules and autophagic membranes. J. Cell Sci. *117*, 4239–4251.

Sullivan, L.C., Clements, C.S., Beddoe, T., Johnson, D., Hoare, H.L., Lin, J., Huyton, T., Hopkins, E.J., Reid, H.H., Wilce, M.C., et al. (2007). The heterodimeric assembly of the CD94-NKG2 receptor family and implications for human leukocyte antigen-E recognition. Immunity *27*, 900–911.

Takeuchi, O., and Akira, S. (2010). Pattern recognition receptors and inflammation. Cell *140*, 805–820.

Watanabe, R., Yoo, Y.C., Hata, K., Mitobe, M., Koike, Y., Nishizawa, M., Garcia, D.M., Nobuchi, Y., Imagawa, H., Yamada, H., and Azuma, I. (1999). Inhibitory effect of trehalose dimycolate (TDM) and its stereoisomeric derivatives, trehalose dicorynomycolates (TDCMs), with low toxicity on lung metastasis of tumour cells in mice. Vaccine *17*, 1484–1492.

Wells, C.A., Salvage-Jones, J.A., Li, X., Hitchens, K., Butcher, S., Murray, R.Z., Beckhouse, A.G., Lo, Y.L., Manzanero, S., Cobbold, C., et al. (2008). The macrophage-inducible C-type lectin, mincle, is an essential component of the innate immune response to *Candida albicans*. J. Immunol. *180*, 7404–7413.

Werninghaus, K., Babiak, A., Gross, O., Hölscher, C., Dietrich, H., Agger, E.M., Mages, J., Mocsai, A., Schoenen, H., Finger, K., et al. (2009). Adjuvanticity of a synthetic cord factor analogue for subunit *Mycobacterium tuberculosis* vaccination requires FcRgamma-Syk-Card9-dependent innate immune activation. J. Exp. Med. *206*, 89–97.

Wines, B.D., Trist, H.M., Ramsland, P.A., and Hogarth, P.M. (2006). A common site of the Fc receptor gamma subunit interacts with the unrelated immunoreceptors FcalphaRI and FcepsilonRI. J. Biol. Chem. *281*, 17108–17113.

Yamaguchi, M., Ogawa, Y., Endo, K., Takeuchi, H., Yasaka, S., Nakamura, S., and Yamamura, Y. (1955). [Experimental formation of tuberculous cavity in rabbit lung. IV. Cavity formation by paraffine oil extract prepared from heat killed tubercle bacilli]. Kekkaku *30*, 521–524.

Yamasaki, S., Ishikawa, E., Kohno, M., and Saito, T. (2004). The quantity and duration of FcRgamma signals determine mast cell degranulation and survival. Blood *103*, 3093–3101.

Yamasaki, S., Ishikawa, E., Sakuma, M., Hara, H., Ogata, K., and Saito, T. (2008). Mincle is an ITAM-coupled activating receptor that senses damaged cells. Nat. Immunol. *9*, 1179–1188.

Yamasaki, S., Matsumoto, M., Takeuchi, O., Matsuzawa, T., Ishikawa, E., Sakuma, M., Tateno, H., Uno, J., Hirabayashi, J., Mikami, Y., et al. (2009). C-type lectin Mincle is an activating receptor for pathogenic fungus, Malassezia. Proc. Natl. Acad. Sci. USA *106*, 1897–1902.

Zelensky, A.N., and Gready, J.E. (2005). The C-type lectin-like domain superfamily. FEBS J. *272*, 6179–6217.

Zhang, J.G., Czabotar, P.E., Policheni, A.N., Caminschi, I., Wan, S.S., Kitsoulis, S., Tullett, K.M., Robin, A.Y., Brammananth, R., van Delft, M.F., et al. (2012). The dendritic cell receptor Clec9A binds damaged cells via exposed actin filaments. Immunity *36*, 646–657.